IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	Tracy A. Willson, et al.	**Examiner:**	Nirmal Singh Basi
Serial No:	10/036,568	**Art Unit:**	1646
Filed:	November 7, 2001	**Docket:**	11373Z
For:	A NOVEL HAEMOPOIETIN RECEPTOR AND GENETIC SEQUENCES ENCODING SAME		

Confirmation No.: 4029

Commissioner for Patents
United States Patent and Trademark Office
Alexandria, Virginia 22313-1450

DECLARATION OF DR. NICOS A. NICOLA UNDER 37 C.F.R. §1.132

Sir:

I, Nicos Anthony Nicola, hereby declare as follows:

1. I am Assistant Director and Head of the Division of Cancer and Haematology of the Walter and Eliza Hall Institute of Medical Research as well as Research Professor in the Department Of Medical biology of the University of Melbourne.

2. I hold a Bachelor of Science (B.Sc.) Degree with Honors in Biochemistry and a Doctorate Degree in Biochemistry from the University of Melbourne. I have conducted research in haemopoiesis and cytokine biology since 1977. I have authored 301 publications in the area of cytokine biology. A true and correct copy of my curriculum vitae is attached hereto as **Exhibit 1**.

3. I have reviewed the above-identified application (hereinafter referred to as "the '568 application") and I am familiar with the subject matter therein. I have also read the Office

Action dated March 27, 2007, issued in the '568 application. I have been asked to review and comment on issues that were raised by the Examiner in the Office Action, particularly with respect to the terms "mature form" and "soluble form" with respect to human NR4 polypeptide. It is my understanding that the Examiner holds the opinion that these terms are unclear and not defined.

4. It is my opinion that that in light of the disclosure in the '568 application, those skilled in the art would understand the terms "mature form" and "soluble form" in reference to a human NR4 polypeptide. Specifically, it is my opinion that based on the information provided in the specification, those skilled in the art would be able to make a reasonable determination of the starting and ending amino acid residues of a "mature form" or "soluble form" of human NR4.

5. My opinion is based on my review of the specification of the '568 application, which expressly describes the mature and soluble forms of the murine NR4, and also describes the close resemblance between the human and murine NR4 proteins.

6. For example, the specification describes various domains of murine NR4 including a signal sequence, an extracellular domain, a transmembrane domain, and a cytoplasmic domain. See Example 6 of the specification (page 37). Figure 1 of the '568 application also depicts the portions of the murine NR4 that represent the signal sequence (amino acids 1-26), and transmembrane segment (amino acids 341-364), respectively. The specification further identifies at page 32, line 25-26, that "A26 or T27" is the predicted first amino acid of the murine "mature protein". Additionally, the specification demonstrates the production of a "soluble" murine NR4 polypeptide, composed of Thr27 to Thr344, on pages 40-41, Example 12. Therefore, the mature form and a soluble form of the murine NR4 are clearly described in the specification of the '568 application.

7. Moreover, the specification discloses in Example 11 (pages 39-40) that SEQ ID NO: 4 is the human homolog of murine NR4, with approximately 75% identity to the murine NR4 at the amino acid level. Figure 7 provides an alignment of the human NR4 sequence with the murine NR4 sequence. It is observed that in Figure 7, Pro27 and Thr28 in human NR4 are aligned on top of Ala26 and Thr27, respectively, of murine NR4 (predicted to be the starting amino acid of the mature form of murine NR4); and that Thr342 in human NR4 is aligned on top of the Threonine residue numbered as 341 in Figure 7 (actually Thr340 in SEQ ID NO: 2 when the gap position in the alignment of Figure 7 is removed) of murine NR4 (predicted to be the ending amino acid of the extracellular region of murine NR4).

8. Therefore, in my opinion, in view of the disclosure in the '568 application, one skilled in the art can make appropriate assessment as to the starting and ending amino acid residues of a "mature form" or "soluble form" of human NR4. Those skilled in the art would also be able to confirm this assessment using methods and techniques known in the art at the relevant time.

9. To my knowledge, a number of methods were available in the art, prior to the original filing of the present application in 1996, for determining the signal sequence and trans-membrane regions of a protein given the amino acid sequence of the protein. In this regard, I refer to the articles attached hereto as **Exhibits 2-5**.

10. **Exhibit 2** ("A new method for predicting signal sequence cleavage sites", Gunnar von Heijne, *Nucleic Acid Research*, 14(11): 4683-4690, 1986) describes a method for predicting the site of cleavage between a signal sequence and the mature protein in 1986 using a weight-matrix approach. This was the most widely used method for predicting the location of the

cleavage site of signal peptides in 1995/1996. This was in fact the common method at the time for predicting signal peptide cleavage points.

11. To my knowledge, the transmembrane region was commonly determined by hydrophobicity analysis. For example, the references as **Exhibits 3-4** describe strategies for predicting transmembrane topology of prokaryotic and eukaryotic membrane proteins. See "Membrane Protein Structure Prediction, Hydrophobicity analysis and the positive inside rule", Gunnar von Heijne, *Journal of Molecular Biology*, 225: 487-494, 1992 (**Exhibit 3**); and "Predicting the Topology of eukaryotic membrane proteins"; Sipos L. and von Heijne G., *Eur J. Biochem*, 213(3): 1333-1340, 1993 (**Exhibit 4**).

12. In addition, the program "Tmpred" makes a prediction of membrane-spanning regions and their orientation of a protein. The program is available at www.chembnet.org/software/TMPRED form.html. The algorithm is based on statistical analysis of TMbase, a database of naturally occurring transmembrane proteins. The prediction is made using a combination of several weight-matrices for scoring. Notably, TMbase was published in 1993. See K. Hofmann & W. Stoffel, "TMbase - A database of membrane spanning protein segments", *Biol. Chem., Hoppe-Seyler*, 374: 166, 1993 (**Exhibit 5**).

13. It is my scientific opinion that the above methods, which were available prior to the filing of the present application, would provide consistent determination as to structures of the "soluble form" and the "mature form" of human NR4. I base this on the observation that once two highly homologous amino acid sequences of two different species are aligned, it is straightforward to translate predictions about one of the sequences to the other. In the '568 application, mouse and human NR4 sequences are aligned such that Ala25 of the mouse sequence is aligned with Ala26 of the human sequence, and amino acids 341-364 of the mouse

NR4 sequence (SEQ ID NO: 2) (labeled as 342-365 in Figure 7 because of the gap created in the alignment) are aligned with amino acids 343-366 of the human NR4 sequence. Since this alignment introduces only two gaps in the mouse compared to human sequence and since the amino acid identity is very high (approximately 75% amino acid identity), this alignment is particularly easy to do.

14. Indeed, the signal sequence prediction programs (SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) Phobius (http://phobius.sbc.su.se/) and Sosui (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html)) each predicted a signal sequence ending at Ala25 for mouse NR4 and Gly25 or Ala26 for human NR4. Similarly application of transmembrane region prediction programs (Phobius, Sosui, TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/)) predict a transmembrane starting sequence between amino acids 341-345 and an ending sequence between 364-365 for mouse NR4 and a transmembrane starting sequence between amino acids 343-347 and an ending sequence between 365-367 for human NR4. These programs thus confirm that a prediction made from the mouse NR4 amino acid sequence translates quite accurately to predictions for the human NR4 sequence at this level of amino acid identity.

15. Further, it is my scientific opinion that through these means available in the art, coupled with the disclosure of the mature and soluble forms of murine NR4, and the similarity and alignment between the murine and human protein sequences, those skilled in the art would have been able to readily determine the signal sequence and trans-membrane regions of the human NR4 protein, thereby determining the structures of the soluble and mature forms of the human NR4 protein, at the time the present application was filed.

16. My opinion is further supported by the findings of Milouex et al. (*FEBS Letters* 401: 163-166, 1997), which is attached hereto as **Exhibit 6**. Milouex et al. characterize the various domains of human IL-13Rα (i.e., human NR4). In particular, the reference describes methods for determining the signal sequence cleavage position and the transmembrane region of human IL-13Rα, as well as the sequences of soluble (containing the extracellular domain) and mature forms of human IL-13Rα. It is noted that the signal sequence of human IL-13Rα (amino acids M1-A26), and the transmembrane segment (amino acids L344 to L367), as indicated in Figure 1 of **Exhibit 6**, are consistent with the murine signal sequence and transmembrane domain proposed in the present application. I observe that the reference was published shortly after the original filing of the present application in 1996, which provides additional support for the notion that those skilled in the art would be able to determine the signal sequence and transmembrane regions of a protein given the sequence of the protein at the relevant time.

17. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____ Dated: Dec 6, 2007
Nicos A. Nicola

Nicos A Nicola AO FAA PhD

Current Positions:	Assistant Director, The Walter and Eliza Hall Institute of Medical Research NHMRC Senior Principal Research Fellow Head, Division of Cancer and Haematology Research Professor of Molecular Haematology, Melbourne University Adjunct Professor, LaTrobe University Honorary Professor, Queensland University
Joined Institute:	1977
Date of Birth:	1 June 1950
Major Research Interests	Haemopoietic Growth Factors/Cytokines Cytokine Receptors and Signalling Leukaemia Development and Treatment

TERTIARY QUALIFICATIONS

1972
BSc (Hons), First Place and First Class Honours, Melbourne University, Biochemistry, supervised by Professor SJ Leach
1976
PhD, Melbourne University, Biochemistry, supervised by Professor SJ Leach

POSITIONS HELD

1971-75
Demonstrator of Biochemistry, University of Melbourne
1975–76
CSIRO Postdoctoral Fellow, Brandeis University, Waltham, Massachusetts
1977–79
Postdoctoral Fellow, The Walter and Eliza Hall Institute of Medical Research, Melbourne.
1979–82
Senior Research Officer, WEHI
1982–86
Research Fellow, Head – JD & L Harris Laboratory for Molecular Regulators, WEHI
1986–91
Senior Research Fellow, WEHI
1991–93
Principal Research Fellow, WEHI
1991–96
Director, Cooperative Research Centre for Cellular Growth Factors
1993–
Senior Principal Research Fellow, WEHI
1996–
Head, Division of Cancer and Haematology, WEHI
Assistant Director, WEHI
1997-
Research Professor of Molecular Haematology, Melbourne University

2002-
Adjunct Professor, Faculty of Science & Technology, LaTrobe University
2004-
Honorary Professor, Queensland University

HONOURS AND AWARDS

1971–75
CSIRO Postgraduate Award, University of Melbourne
1975
CSIRO Postdoctoral Fellowship
1977
Queen Elizabeth II Postdoctoral Fellowship
1986
Gottschalk Medal of the Australian Academy of Science
1989–90
Volunteer Units Research Award of the Anti-Cancer Council of Victoria
1991
Pharmacia-LKB Biotechnology Medal of the Australian Society for Biochemistry and Molecular Biology
1993
Wellcome Australia Medal
1996
Fellow of the Australian Academy of Science
Amgen Australia Prize
1998
Governing Council Member, International Molecular Biology Network for Asia and the Pacific Rim
1999
Austin Doyle Lecturer, High Blood Pressure Research Council of Australia, AGM, Melbourne
Barbara Ells Lecturer, Victor Chang Cardiac Research Institute, Sydney
2001
ISI Australian Citation Laureate Award (11 papers published between 1981-98 which were in the 200 most cited papers in their field internationally).
2003
Prime Minister's Centenary Medal
Honorary Professorship, University of Queensland
2005
Officer in the General Division of the Order of Australia

PROFESSIONAL ACTIVITIES

Scholarly

Editorial Boards

1990–1992
Experimental Hematology
1990-
Stem Cells
Growth Factors
1991
Current Opinion in Hematology
1994–
International Journal of Hematology
1994–99
Journal of Cell Science

1994–95
Trends in Biochemical Sciences
1995–99
Cytokine and Growth Factor Reviews
1997-
Cytokines On Line
1998-
Molecular Cell Biology Research Communications
1999-
Experimental Hematology
2007-
Technology Transfer Tactics
Open Biotechnology

Conference Presentations

1989
Invited Speaker, Bone Biology Workshop, New Jersey
Invited Speaker, Sapporo Cancer Seminar, Sapporo
Invited Speaker, Growth Factors International Symposium IP, Kobe
Lecture, Kyoto Prefectural University of Medicine, Kyoto
Invited Speaker, ASI/ASMR National Scientific Conference, Adelaide

1990
Invited Speaker, Lorne Protein Conference, Lorne
Invited Speaker, Third International Workshop on Cells and Cytokines in Bone and Cartilage, Davos
Invited Speaker, Hemopoietic Growth Factors Conference, Tokyo
Invited Speaker, AACR Special Conference on Chromosomal and Growth Factor Abnormalities in Leukemia, Cape Cod
Invited Speaker, Hanson Symposium, Adelaide
Invited Speaker, Combined International Society of Hematology, Boston
American Society of Hematology Annual Meeting, Boston
Lecture, Dana Farber Cancer Institute, Boston

1991
Invited Speaker, Lorne Protein Conference, Lorne
Invited Speaker, Australian Endocrine Society Annual Meeting, Lake Hume
Invited Speaker, UCLA Conference Cytokines and their Receptors, Keystone
Award Recipient, Australian Society for Biochemistry and Molecular Biology, Annual Meeting, Canberra
Invited Speaker, Blood Cell Growth Factors: Their Present and Future use in Hematology and Oncology, Beijing
Invited Speaker, Arolla Workshop: From Receptor to Gene, Arolla
Invited Speaker, CIBA Symposium 167, Polyfunctional Cytokines – IL-6 and LIF, London
Invited Speaker, 15th Bristol-Myer Squibb Symposium on Cancer Research, Seattle

1992
CRC Directors' Meeting, Sydney
Invited Speaker, International Society of Experimental Hematology, Annual Meeting, Rhode Island
Lecture, Pharmacia-LKB Biotechnology Medal Lecture, University of Sydney, Sydney
Lecture, Pharmacia-LKB Biotechnology Medal Lecture, Queensland University, Queensland
Invited Speaker, Hanson Symposium, Adelaide
Invited Speaker, Australian & New Zealand Society of Immunology, Annual Meeting, Auckland

1993

Invited Speaker, Bioscience Forum 93, Osaka
Assigner, NH&MRC Assigners' Panel, Canberra
CRC Director – CRC Directors' Meeting, Brisbane
Invited Speaker, March Foundation Symposium, Madrid
Discussant, Sandoz Clinical Trial Meeting, Basel
Invited Speaker, Australian Society for Medical Research Annual Meeting, Adelaide
Session Chairman and Invited Speaker, Joint Meeting of the Australasian Society for Immunology and International Congress of the Society for Leukocyte Biology, Sydney
Invited Speaker, Association of Regulatory and Clinical Scientists Conference, Brisbane

1994

Invited Speaker, Training Course, UICC (International Union Against Cancer), Anti-Cancer Council of Victoria, Ludwig Institute for Cancer Research, Melbourne
Invited Speaker, Growth Factor Session of Neurosciences Towards 2000 Conference, Melbourne
Invited Speaker, Transfusion Medicine in Obstetrics and Neonatology Conference, Melbourne
Invited Speaker, Growth Factors and Genes in Myogenesis, St Vincent's Medical Research Institute, Melbourne
Invited Speaker, Seminar, Cooperative Research Centre for Biopharmaceuticals, The Garvan Institute of Medical Research, Sydney
Invited Speaker, New York Academy of Sciences Conference on Receptor Activation, Orlando
Invited Speaker, Hanson Symposium, Hanson Centre for Cancer Research, Adelaide
Invited Speaker, Australian Society for Medical Research Annual Conference, World Trade Centre, Melbourne

1995

Invited Speaker, 1st Annual Curtin Conference, Cell Signalling: From Membrane to Nucleus, Canberra
Invited Speaker, Volunteer Unit's Annual Meeting, Anti-Cancer Council of Victoria, Melbourne
Assigner, National Health and Medical Research Council Assigners' Panel, Canberra
CRC Director, Cooperative Research Centre Directors' Meeting, Melbourne
Invited Speaker, Seminar, Monash Medical Centre, Melbourne
Invited Speaker, Horizons of Science Forum Conference, University of Technology, Sydney
Speaker, International Society of Experimental Hematology – Annual Meeting, Düsseldorf
Invited Speaker, 7th FAOB Conference, Sydney

1996

Invited Speaker, Lorne Cancer Conference, Lorne
Invited Speaker, CIBA Conference, Molecular Basis of Cellular Defence Mechanisms, Melbourne
Invited Speaker, Cytokines in Bone Marrow Transplantation, Sydney
NH&MRC Assigners' Panel, Canberra
CRC Directors' Meeting, Sydney
Invited Speaker, Cytokine Receptors and Signal Transduction, Annual Inflammation Symposium, Sydney
Invited Speaker, Interferons and Cytokines, Saudi Arabia
Invited Speaker, 3rd Symposium on Haemopoietic Growth Factors, Tokyo
Invited participant, AMRAD-CHUGAI Scientific Meeting, Tsukuba and Gotemba
Invited Speaker, International Symposium for Stem Cell Regulation, Tokyo
Invited Speaker, Combined ASBMB/ASPP. Annual Scientific Meeting, Canberra
Invited Speaker, Australian Vascular Biology Society, 4th Annual Scientific Meeting, Marysville
Scientific Committee Meeting, 1st Australian Health Industry Expo, Sydney
Invited Seminar, Institute of Reproduction and Development, Monash Medical Centre
Invited Speaker, CRC-CGF Workshop on Cytosensor Technology
Invited Speaker, Hanson Symposium "Molecular Mechanisms of Oncogenesis," Adelaide
Invited Speaker, Satellite Symposium on Cell Signalling, Adelaide

1997

Invited Seminar, Microbiology Dept, Melbourne University
Assigners' Panel, NHMRC, Canberra
Induction into Australian Academy of Science, Canberra
Invited Seminar, QIMR, Brisbane
Invited participant, 1st Meeting of International Molecular Biology Network and 4th IMSUT-IMBG Symposium, Tokyo
Invited Speaker, IMSUT, Tokyo
Invited Speaker, Chugai Institute of Molecular Medicine, Tsukuba
Invited Speaker, 'From the Laboratory to the Clinic,' Trinity College, Oxford
Invited Speaker, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford
Invited Participant, 'Meeting to discuss development of a not-for-profit company to develop tropical disease products,' The Wellcome Trust, London
Plenary Speaker, 'The Myelodysplastic Syndromes,' The McCarthy Foundation, Detroit
Regional Grants Interview Committee, NHMRC, Perth
Invited Chairman, Chugai Scientific Symposium, Gotemba, Japan

1998

Invited Speaker, DNA Science Workshop for Secondary School Teachers, Melbourne
Invited Speaker, 'Reversible Associations in Structural and Molecular Biology,' RASMB, Melbourne
Plenary Speaker, '11th Symposium of Molecular Biology of Hematopoiesis,' Bormio, Italy
Plenary Speaker, '10th Anniversary Meeting of the British Cytokine group,' London
Invited Speaker, 'Current Issues in Hemopoiesis,' ASH/ASBT Annual Meeting, Sydney
Invited Speaker, 'Signal Transduction and Subcellular Compartmentalization,' Victor Harbour, South Australia
Invited Speaker, 3rd Australian Peptide Conference, Laguna Quays, Queensland
Invited Lecture, Centre for Immunology, University NSW and St Vincent's Hospital, Sydney

1999

Plenary Speaker, International Society for Experimental Hematology Annual Meeting, Monte Carlo
Plenary Speaker, 64th Cold Spring Harbor Symposium 'Signaling and Gene Expression in the Immune System', Cold Spring Harbor, New York
Invited Speaker, 1st Meeting of the Governing Council of A-IMBN, Tsukuba, Japan
Invited Speaker, 1st A-IMBN Conference 'Frontiers in Molecular Biology', Singapore
Plenary Speaker, French Society of Immunology Annual Meeting, Lille, France
Invited Lecture, St Vincent's Institute of Medical Research, Melbourne
Invited Participant, Baker Institute Retreat, Daylesford
Invited Lecture, Ivanhoe Grammar School
Austin Doyle Lecturer, High Blood Pressure Research Council of Australia, AGM, Melbourne

2000

Invited Speaker, Brisbane BioIndustry Breakfast, Brisbane.
Invited Discussant, National Innovation Summit, Melbourne.
Invited Speaker, 2nd International Workshop on Cytokine Signaling ,Aachen, Germany.
Invited Speaker, 10th Queenstown Molecular Biology Meeting, Queenstown, NZ.
Invited Speaker and Chairperson, International Congress on Differentiation and Cell Biology, Gold Coast
Invited Speaker, Kobe Symposium:GP130 Cytokines in Health and Disease, Kobe.
Invited Speaker, 'Molecular Biology and Medicine on Infectious and Immunological Diseases', Osaka
Invited Speaker, Monash Institute for Reproduction and Development, Melbourne
Barbara Ells Lecturer, Victor Chang Cardiac Research Institute, Sydney
Invited Speaker, 3rd Joint Meeting ICS/ISICR, Amsterdam
Invited Speaker, Biotech 2000 Conference, Victor Chang Cardiac Research Institute, Sydney

2001

Invited participant, A-IMBN symposium and Governing Council Meeting, Tokyo
Awardee, ISI Symposium 'Honouring Excellence in Australian Research' AAS Dome, Canberra
Invited Plenary speaker, ISICR Meeting, Cleveland,Ohio
Invited Speaker, 'Comprehensive Cancer Research Centre: Biology to Bedside' Brisbane Aug 29-30
Invited Speaker, South East Asian Biomolecular Research Training Programme Workshop, Couran Cove, Qld
Invited Speaker, Monash University Biochemistry Department Seminar Series
Invited Speaker, Opening Conference Rommelare Institute, Ghent Sept 13-14
Invited Speaker, A-IMBN symposium, Taiwan
Invited Speaker, RMIT Health Sciences, Bundoora

2002

Invited Plenary Speaker, Gordon Conference on Neurobiology, Hong Kong (Jun)
Invited Speaker, Keystone Meeting, Keystone, Colorado, Jan 2002
Invited Plenary Speaker, COMBIO 2002, Sydney (Sept)
Keynote Speaker, Licensing Executives Soc. Of Aust and NZ annual meeting, Werribee (Apr)
Invited Speaker, Institute of Knowledge Development, Managing Business Issues Series, Melb (Apr)
Invited Speaker, 2nd Awaji Intnl Forum on Infection and Immunity, Awaji (Aug)
Invited Speaker, Health and Medical Research Conference, Melbourne (Nov)
Invited Speaker, Prince Henry's Institute, Melbourne (Jul)
Invited Speaker, QIMR Annual Conference, Gold Coast (Sept)
Invited Speaker, Combined ASI/SCIL Conference, Brisbane (Dec)

2003

Invited Speaker and Chairman, Joint Session Lorne Protein and Cancer Conferences, Lorne (Feb)
Invited Speaker and Chairman, Japan/Australia Cancer Meeting, Healesville (Feb)
Invited Speaker, Hunter Valley Cell Biology Meeting (Apr)
Invited Speaker, Opening conference, IMCB, Brisbane (May)
Invited participant, National Centre in HIV Virology Research, Strategic Planning Day (Feb)
Invited Speaker and Chairman, International Conference on Cellular Engineering, Bondi (Aug)
Plenary Speaker, NSW ASMR conference, Sydney (June)
Invited Plenary Speaker, International Society for Interferon and Cytokine Research Annual Meeting, Cairns (Oct)

2004

Invited Plenary Speaker, New Era for Gene Medicine, Tokyo (Mar)
Speaker, NHMRC Program Grant Consultations, Sydney, Brisbane, Adelaide, Canberra, Melbourne (Jul)
Invited Plenary Speaker and Chair, Haematology Society of A&NZ, Melbourne (Oct)
Invited Speaker, Symposium of Australian Academy of Technological Sciences and Engineering, Adelaide (Nov)
Invited Speaker, ASMR annual conference, Sydney (Nov)

2005

Invited Speaker, IMB conference (April)
Invited Speaker, Medical Research Week, Melbourne (Jul)
Invited Speaker, 7th World Congress on Inflammation, Melbourne (Aug).
Invited Speaker, Japan Australia Collaboration in Biomedicine, Aichi, Japan (Sept)
Invited Seminar, Baker Institute (Sept)
Invited Speaker, 'Signalling Networks', Barossa Valley (Nov).

2006

Invited Speaker, Garvan International Fellow Symposium, Sydney (May).
Invited Speaker, Commercialising Research Conference, Melbourne, (Oct).

2007

Invited Seminar, Hanson Cancer Centre, Adelaide (May)
Invited Plenary Speaker, 'Congenital Bone Marrow Failure Syndromes', Hannover, Germany (Sept)

Invited Speaker, 3rd Barossa Meeting 'Signalling Systems' Barossa (Nov)

2008
Invited Plenary Lecturer, Sydney Cancer Conference 2008, Sydney (Jul)

Administrative

External Committees

1991–96
Management and Scientific Committees for CRC-Cellular Growth Factors
1992–96
NH&MRC Assigners' Panel
CRC-Medical Science and Technology Section Committee
1994–95
Appointments and Promotion Committee, Ludwig Institute, Melbourne
1994–2001
Appointments and Promotions Committee, Baker Institute, Melbourne
1995
Australia Prize Selection Committee
1995–
Scientific Advisory Board, Hipple Cancer Center, Ohio
1997
Chairman, Scientific Committee, CRC-Cellular Growth Factors
Research Committee, NH&MRC
RGIC, NH&MRC
Royal Adelaide Hospital Campus Review Committee
Task Force Committee, International Molecular Biology Network
IMBN Expert Commission for Molecular Biology Needs for Asia and the Pacific Rim
1998-
Medical Review Committee – J.P. McArthy Foundation, Detroit, Michigan
Board, Prince Henry's Medical Research Institute
Research Committee, NH&MRC
Governing Council of A-IMBN
Scientific Committee, International Society for Experimental Hematology Annual Meeting 1999
1999-2002
Australian Academy of Science, Sectional Committee 8, Biochemistry, Molecular Biology and Immunology
Scientific Committee, International Society for Experimental Hematology Annual Meeting 2000
Biotechnology Consultative Group (BIOCOG) reporting to the five federal ministers.
Biotechnology Strategic Plan Steering Committee, advising Department of State and Regional Development, Victorian Government.
2000-2003
Research Committee (RC) of NHMRC
RC executive
Chair, Industry Committee of RC
2001-
Scientific Advisory Board, Institute of Molecular Bioscience, Qld.
Working Group 3, Pharmaceutical Manufacturers' Association Action Agenda
2002-
Scientific Advisory Board, Bio21

Scientific Advisory Board, CRC Chronic Inflammatory Diseases
Scientific Advisory Board, Queensland Institute for Medical Research
2003-2006
Research Committee (RC) of NHMRC
RC executive
Chair, Programs Committee of RC
2005
Australian Academy of Science, Sectional Committee 8
Australian Academy of Science, Boden selection committee
NHMRC MORIA Working Group
2006
DEST Research Quality Framework Scoping Workshop
Scientific Review Committee, Telethon Institute for Child Health Research, WA.
Scientific Review Committee, Centre for Immunology and Cancer Research, Qld
Scientific Review Committee, CRC for Chronic Inflammatory Diseases, Vic
Review Committee, Cancer Council of Victoria Venture Grants
Cancer Centre Advisory Group
Peter Mac Relocation Project Team
2007
DEST RQF Taskforce Discipline workshop, ACT
Scientific Advisory Board, Australian Stem Cell Centre, Melbourne
Chair, Development Grants Committee, NHMRC
Advisory Board, Institute for Molecular Bioscience
Review Committee, Cancer Council of Victoria Venture Grants
Internal Audit Committee, ASCC
Australian Academy of Science, Boden selection committee

WEHI Committees

1990–96
Chairman, Internal Finance and Advisory Committee
Library Committee
Faculty Committee
1992–96
Unit Heads Committee
1997-
Chairman, Technology Advisory Committee
Parkville Bioinformatics Committee
Senior Scientific Advisory Committee
Senior Executive Committee
Senior Faculty Committee
Faculty Commercialization Committee
2006-
Board Commercialisation Committee
Board New Building Sub-committee
WEHI Extension Executive Committee
WEHI Western Expansion Steering Committee
WEHI Western Expansion Executive Planning Team
WEHI Appointments and Promotions Committee

Industry Consulting

1990-
AMRAD Corporation, Melbourne

1995-200
Chugai Corporation, Japan
2000-
co-founder, Quintessential Sciences Inc.
co-founder and SAB, Murigen Inc.
2004
Biota
2005-2006
CSL
2007
Australian Stem Cell Centre

Public Activities

1995
Horizons of Science Forum, Sydney
1995-
Submissions to Industry Commission Reports on Research and Development, Innovation, Health andMedical Research Review and CRC programme
1999.
Lecture on Biotechnology, Ivanhoe Grammar School
Lecture on Biotechnology, Federal Dept. Health and Aged Care
2000.
Invited Speaker, Brisbane BioIndustry Breakfast, Brisbane.
Invited Discussant, National Innovation Summit, Melbourne
Chairman, Biotechnology Australia Intellectual Property Symposium, Adelaide
Invited Speaker, RICH Symposium of ASMR
Invited Speaker, BIOTECH 2000, Sydney
2002
Invited speaker, Institute of Knowledge Development, Business Issues Breakfast Series, Melbourne
Business Breakfast, Biotechnology Course Consultations, Box Hill TAFE
2003
Invited participant, NIH IP Policy Contact Group, Canberra
Invited participant, National Centre in HIV Virology Research, Strategic Planning Day (Feb)
2006
Facilitator, Victor Chang Cardiac Research Institute Strategic Planning Day

TEACHING AND SUPERVISION 1990–1996

Tertiary

1987–91
DJ Hilton, PhD, Characterization of LIF and its Cell Surface Receptor
1992–95
WJ McKinstry, PhD, Molecular Analysis of Factors Active on Haemopoietic Stem Cells
1993–96
A Smith, PhD, Cellular Signalling by the GM-CSF Receptor β-chain
1996-98
Kelly Maxwell, PhD
1997.
Ruth Freeman, BSc (Hons)
2001-2005
Ruth Columbus, PhD
2002-2006
Seth Masters, PhD
David DeSouza (BSc Hons)
Marlyse DeBrincat, PhD
2006-
Anjana Chakravorty, PhD

Post-doctoral

1990–
DJ Hilton
P Lock
Y Zhang
J-G Zhang
R Starr
C McFarlane
S Nicholson
M Baca
A Roberts
D Krebs

GRANTS AND CONTRACTS AWARDED 1990–

1990–
~$100,000 pa, NIH 2ROI-CA-22556, Differentiation of Granulocytes and Macrophages
$300,000 pa, AMRAD, Haemopoietic Growth Factors
$50,000 pa, JD & L Harris Trust, General
1991–2004
$30,000 pa, Philip Bushell Trust Equipment Grant
$2m pa, Cooperative Research Centres Grant, Growth Factors
1996-
$1.5m pa AMRAD grants (SOCS, LIF/IL-6, NR4,NR6)
2001-2005
US200,000pa NIH 2ROI-CA-22556, Differentiation of Granulocytes and Macrophages

2003-2007
$2.75m pa NHMRC New Program Grant (CIA)
2003
AMGEN research grant US$75,000

2005-2009
US$250,000 pa. NIH 2ROI-CA-22556, Differentiation of Granulocytes and Macrophages, **Merit Award**
2005-2006
Australian Stem Cell Centre Grant. $175,000 pa.
2007-2011
$3.25m pa NHMRC Program Grant 461219 (CIA)

PUBLICATIONS

Refereed Primary Publications

1975

1. Appleby CA, NICOLA NA, Hurrel JGR, Leach SJ. Characterization and improved separation of soybean leghemoglobins. Biochem 14: 4444-4450, 1975
2. NICOLA NA, Minasian EM, Appleby CA, Leach SJ. Circular Dichroism studies of myoglobin and leghemoglobin. Biochem 14: 5141-5149, 1975

1976

3. Hurrel JGR, NICOLA NA, Broughton WJ, Dilworth MJ, Minasian EM, Leach SJ. Comparative structural and immunochemical properties of leghemoglobins. Eur J Biochem 66: 389-399, 1976
4. NICOLA NA, Leach SJ. Interpretations and applications of thermal difference spectra of proteins. Int J Prot Pept Res 8: 393-415, 1976

1977

5. NICOLA NA, Leach SJ. The structural basis of heme reactivity in myoglobin and leghemoglobin: Thermal difference spectra. Biochem 16: 50-58, 1977
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1978

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A new method for predicting signal sequence cleavage sites

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ABSTRACT

A new method for identifying secretory signal sequences and for predicting the site of cleavage between a signal sequence and the mature exported protein is described. The predictive accuracy is estimated to be around 75-80% for both prokaryotic and eukaryotic proteins.

INTRODUCTION

The transient N-terminal signal sequence found on most secretory proteins serves to initiate export across the inner membrane (in prokaryotes) or the endoplasmic reticulum (in eukaryotes). Three structurally and, possibly, functionally distinct regions have been identified as the basic building-blocks of a secretory signal sequence: a basic N-terminal region (n-region), a central hydrophobic region (h-region), and a more polar C-terminal region (c-region) (1). The structural determinants for cleavage of the signal sequence from the mature protein once export is under way seems to reside in the n- and h-regions, with positions -3 and -1 relative to the cleavage site being the most important ones (2,3). Indeed, this "(-3,-1)-rule" has been used quite successfully to predict the most likely site of cleavage directly from the primary sequence (2).

In view of the great interest in secretory proteins and the fact that most such proteins are known only from their DNA sequence, it is important to assess and, if possible, to improve upon the predictive accuracy of the original method. In this paper, I present a new scheme based on a weight-matrix approach that can be expected to give correct predictions about 75-80% of the time when applied to new sequences (both prokaryotic and eukaryotic). This represents a substantial gain over the old method, which is shown to be around 65% and 45% accurate for eukaryotic and prokaryotic proteins, respectively.

METHODS

161 eukaryotic and 36 prokaryotic non-homologous signal sequences with known cleavage sites were chosen from my collection of signal sequences totalling at the present time some 450 eukaryotic and 80 prokaryotic entries. The prokaryotic sample did not include any sequences known to be cleaved by the lipoprotein signal peptidase (signal peptidase II) (4).

Weight-matrices W(a,i) (see below) were calculated from the observed amino acid counts in each position, N(a,i), (i.e. the number of residues of type a in position i) with all sequences aligned from their known site of cleavage between positions -1 and +1, by first dividing all counts by their respective expected abundance in proteins in general, <N(a)> (Tables 1 & 2, last column), and then taking the natural logarithms of these quotients: $W(a,i) = \ln(N(a,i)/\langle N(a)\rangle)$. To correct for the limited size of the data base, all zero-elements in the amino acid count matrices were put equal to one before the divison. Zero-counts in positions -3 and -1 were treated differently: they were also put equal to one, but then divided by the total number of sequences in the sample, N, rather than the expected number of residues, e.g. $W(a,-1) = \ln(1/N)$ if $N(a,-1) = 0$.

The most probable cleavage site was identified by scanning the sequence in question with the appropriate weight-matrix and summing the weights for each position, i.e. $S(i) = W(a_{i-p},i-p) + W(a_{i-p+1},i-p+1) + \ldots + W(a_{i+q},i+q)$ where the summation window extends from position i-p to i+q. The predicted cleavage site j is the one with the highest S-value, $S(j) = \max[S(i);\ i=1-p,\ldots,L-q]$, where L is the length of the sequence analyzed. As shown below, maximum predictive accuracy was obtained for p=-12 and q=2.

RESULTS

The (-3,-1)-rule

Based on previous statistics (2), acceptable cleavage sites were suggested to conform to the following rules: the residue in position -1 must be small, i.e. either Ala, Ser, Gly, Cys, Thr, or Gln; the residue in position -3 must not be aromatic (Phe, His, Tyr, Trp), charged (Asp, Glu, Lys, Arg), or large and polar (Asn, Gln). Further, it was suggested that Pro must be absent from positions -3 through +1. The new amino acid counts presented in Tables 1 & 2 are based on more than twice as many sequences; nevertheless, the (-3,-1)-rule is seen to hold remarkably well. The only exceptions found to date among eukaryotic proteins are one sequence with Leu in -1, one with Pro in -2, and three with Pro in -1. Thus, barring sequencing errors, we must

Table 1 **Amino acid counts for eukaryotic signal sequences**
The average composition (last column) is from Ref.(10)

	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	Expected
A	16	13	14	15	20	18	18	17	25	15	47	6	80	18	6	14.5
C	3	6	9	7	9	14	6	8	5	6	19	3	9	8	3	4.5
D	0	0	0	0	0	0	0	0	5	3	0	5	0	10	11	8.9
E	0	0	0	1	0	0	0	0	3	7	0	7	0	13	14	10.0
F	13	9	11	11	6	7	18	13	4	5	0	13	0	6	4	5.6
G	4	4	3	6	3	13	3	2	19	34	5	7	39	10	7	12.1
H	0	0	0	0	0	1	1	0	5	0	0	6	0	4	2	3.4
I	15	15	8	6	11	5	4	8	5	1	10	5	0	8	7	7.4
K	0	0	0	1	0	0	1	0	0	4	0	2	0	11	9	11.3
L	71	68	72	79	78	45	64	49	10	23	8	20	1	8	4	12.1
M	0	3	7	4	1	6	2	2	0	0	0	1	0	1	2	2.7
N	0	1	0	1	1	0	0	0	3	3	0	10	0	4	7	7.1
P	2	0	2	0	0	4	1	8	20	14	0	1	3	0	22	7.4
Q	0	0	0	1	0	6	1	0	10	8	0	18	3	19	10	6.3
R	2	0	0	0	0	1	0	0	7	4	0	15	0	12	9	7.6
S	9	3	8	6	13	10	15	16	26	11	23	17	20	15	10	11.4
T	2	10	5	4	5	13	7	7	12	6	17	8	6	3	10	9.7
V	20	25	15	18	13	15	11	27	0	12	32	3	0	8	17	11.1
W	4	3	3	1	1	2	6	3	1	3	0	9	0	2	0	1.8
Y	0	1	4	0	0	1	3	1	1	2	0	5	0	1	7	5.6

admit the possibility that residues other than the classical (-3,-1)-kinds can be used in position -1, but only when no better cleavage site is available in the vicinity (this is true for all five exceptions).

A few other points can also be made. First, the constraints on the prokaryotic sequences in the (-3,-1)-region seem even stronger than for the eukaryotic ones: only Ala, Gly, Ser and Thr have been found in -1, and only Ala, Gly, Leu, Ser, Thr, and Val in -3. Second, Leu is abundant in the prokaryotic sample up to and including position -8, but its incidence drops precipitously in position -7, where it is replaced by the likewise hydrophobic but less strongly helix-inducing residues Val and Phe. Only from position -6 do we find predominantely polar residues. Finally, there is a notable imbalance between the basic residues Arg and Lys in the c-region of the eukaryotic signal sequences, with 26 Arg and only 6 Lys (Arg/Lys = 4.3). This is in sharp contrast to the n-region where Arg/Lys = 66/72 = 0.9 and to proteins in general where the expected ratio is 0.6 (Table 1, last column).

Table 2 **Amino acid counts for prokaryotic signal sequences**
The average composition (last column) is from Ref.(10)

	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	Expected
A	10	8	8	9	6	7	5	6	7	7	24	2	31	18	4	3.2
C	1	0	0	1	1	0	0	1	1	0	0	0	0	0	0	1.0
D	0	0	0	0	0	0	0	0	0	0	0	0	0	2	8	2.0
E	0	0	0	0	0	0	0	0	0	0	0	1	0	4	8	2.2
F	2	4	3	4	1	1	8	0	4	1	0	7	0	1	0	1.3
G	4	2	2	2	3	5	2	4	2	2	0	2	2	1	0	2.7
H	0	0	1	0	0	0	0	1	1	0	0	7	0	1	0	0.8
I	3	1	5	1	5	0	1	3	0	0	0	0	0	0	2	1.7
K	0	0	0	0	0	0	0	0	0	1	0	2	0	3	0	2.5
L	8	11	9	8	9	13	1	0	2	2	1	2	0	0	1	2.7
M	0	2	1	1	3	2	3	0	1	2	0	4	0	0	1	0.6
N	0	0	0	0	0	0	0	1	1	1	0	3	0	1	4	1.6
P	0	1	1	1	1	1	2	3	5	2	0	0	0	0	5	1.7
Q	0	0	0	0	0	0	0	0	2	2	0	3	0	0	1	1.4
R	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1.7
S	1	0	1	4	4	1	5	15	5	8	5	2	2	0	0	2.6
T	2	0	4	2	2	2	2	2	5	1	3	0	1	1	2	2.2
V	5	7	1	3	1	4	7	0	0	4	3	0	0	2	0	2.5
W	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0.4
Y	0	0	0	0	0	0	0	0	0	3	0	1	0	0	0	1.3

Construction of weight-matrices

Weight-matrix methods have been used for a number of years to locate signals in nucleic acid sequences (see (5) for a thorough discussion). Their use for pattern recognition in protein sequences requires a larger data base (20 amino acids rather than 4 bases must be scored for in each position), but is no different in principle. Basically, one converts the observed number of each kind of residue in each position in a sample of aligned "signals" into a measure of the probability of finding that particular kind of residue in that particular position - the probability weight-matrix - by a suitable normalization. Then, any new sequence can be scanned by a moving window (looking up the respective probabilities in the weight-matrix and multiplying together for each position of the window) to get a measure of the fit to the sample used in the construction of the weight-matrix. The highest-scoring window-position is then taken as the prediction for the location of the signal, if the score is above some minimum value.

To score for possible signal sequence function, and to locate the most probable cleavage site in a putative signal sequence, weight-matrices for prokaryotic and eukaryotic signal sequences were constructed as follows. The raw amino acid counts for the two samples (Tables 1 & 2) were divided by the expected number $\langle N(a) \rangle$ of each kind of residue given amino acid frequencies as in soluble proteins in general (last columns). Except for positions -3 and -1 relative to the cleavage site, all matrix elements with zero counts were normalized as $1/\langle N(a) \rangle$. For positions -3 and -1, where there is good reason both from previous statistical and experimental studies to believe that only a subset of all residues are allowed (2,6), the more stringent normalization 1/N was used for the zero-count elements (where N is the total number of sequences in the sample). The final weight-matrix was obtained by taking the natural logarithms of the normalized values, thus reducing the ensuing probability calculations to summations rather than multiplications of the weight-matrix elements.

Assessment of the predictive accuracy

When the two weight-matrices were used to predict the cleavage sites in the samples used in their construction, virtually all sites were correctly identified (87% in the eukaryotic sample, 100% in the prokaryotic sample). However, these sequences are at an advantage relative to sequences not included in the matrix: when correctly aligned with the weight-matrix, all residues in a sequence included in the weight-matrix sample will correspond to a count, and a spuriously high predictive accuracy will be found.

To avoid this problem, the eukaryotic sample was divided into 7 subsamples, each of 23 sequences. For each subsample, the remaining 138 sequences were used to construct a new weight-matrix, and this matrix was then applied to the subsample. Similarly, the prokaryotic sample was divided into 4 subsamples, each of 9 sequences. All subsequent calculations were carried out by summing the results for the subsamples.

Weight-matrices including positions -15 to +5 were first used to determine the effect of ignoring residues at either end in the predictions. It was found that positions -13 to +2 were sufficient to obtain maximal predictive accuracy (for the prokaryotic sample, positions -5 to +2 were sufficient but the full -13 to +2 range was used nevertheless): with this choice, 125 out of 161 eukaryotic and 32 out of 36 prokaryotic cleavage sites (78% and 89%) were correctly identified with a standard deviation of about ±10% in each case. For an additional 19 eukaryotic and 2 prokaryotic sequences, the correct site had the second-highest score. These values should

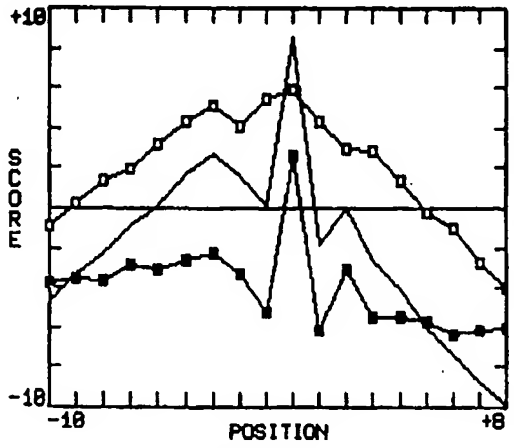

Figure 1 Average h- and c-region scores as a function of the position of the moving window. Open squares: h-region; solid squares: c-region; full line: total score.

be compared with the predictive accuracy of the older method (as implemented in a program kindly communicated by Dr. H.S. Ip, Rockefeller University). When this method was applied to the 121 sequences in the eukaryotic sample that were not included in the original statistics (2), 77/121 (64%) of the known cleavage sites were correctly identified, and only 17/36 (47%) of the prokaryotic ones were found.

With -13 to +2 weight-matrices, the contribution to the overall success from individual positions was also investigated. Only positions -3 and -1 had any strong impact; when one or the other was left out in the calculations the percentage of correctly identified eukaryotic sites dropped to 61% and 53%, respectively (81% and 69% for the prokaryotic sample).

As has been shown previously (1,7), residues -13 to -6 correspond to the h-region in the "average" eukaryotic signal sequence, residues -5 to -1 correspond to the c-region, and residues +1 and +2 seem to be selected such that few alternative cleavage sites should exist in the vicinity of the correct one (i.e. residues -5 to +2 can be included in an extended c-region). Thus, it is possible to calculate the scores for the h- and c-regions separately by summing the contributions from positions -13 to -6 and -5 to +2, respectively. As shown in Fig.1, the average h-region score for the eukaryotic sample increases slowly as the window is moved up to position -1 (the known cleavage site), and then decreases. The average c-region score

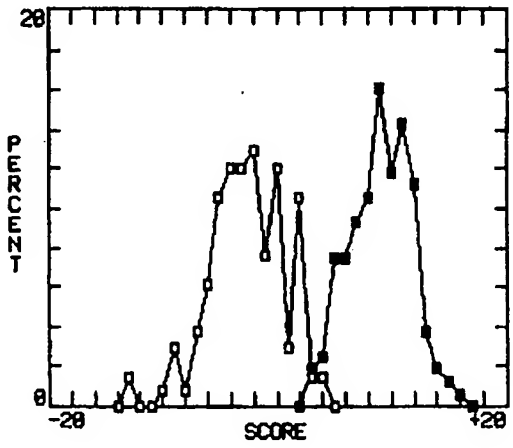

Figure 2 Distribution of maximum scores for signal sequences and cytosolic proteins. Open squares: cytosolic proteins; solid squares: signal sequences.

shows a more dramatic behaviour, with a pronounced peak in position -1 and troughs in positions -2 and +1, reflecting the match to the (-3,-1)-pattern and the tendency to have residues in position -2 that do not fit this pattern (see Tables 1 & 2). Similar curves are obtained for the prokaryotic sample (not shown).

Interestingly, 35 out of the 36 erroneous predictions for the eukaryotic sequences fall on the N-terminal side of the correct cleavage site, mostly in the region -6 to -3 (30/36). About half of these result from matches with a higher score in the h-region but a lower one in the c-region than calculated for the correct site, whereas only 6 out of 36 have higher c- and lower h-region scores than the correct site. I have thus tried to improve the predictive accuracy in various ways, e.g. by multiplying the -3 and -1 weigths or the whole c-region score by an extra factor, or by allowing a small variation in the distance between the h- and c-regions, but have not been able to obtain more than marginal improvements on the order of 2-4% in the overall success-rate.

The method described here not only allows prediction of the most likely cleavage site in new signal sequences, it also makes it possible to discriminate quite efficiently between putative signal sequences and the N-terminal regions of cytosolic proteins. The distribution of maximum scores for the eukaryotic signal sequences is shown in Fig.2, together with the

corresponding distribution obtained for a sample of 132 40-residues long N-teminal regions of cytosolic eukaryotic proteins (8). Only 3/161 (2%) of the signal sequences have maximum scores < 3.5; conversely, only 2/132 (2%) of the cytosolic sequences have maximum scores > 3.5. This level of discrimination compares favourably with that obtained with a recently published signal-sequence detecting algorithm (9).

DISCUSSION

Using a standard weight-matrix approach easily implemented even on a micro-computer, it is possible to set up a prediction method that (i) provides a clean discrimination between signal sequences and the N-terminal region in cytosolic proteins, and (ii) can be expected to identify the correct cleavage site 75-80% of the time when applied to new sequences not included in the data base (both prokaryotic and eukaryotic). This represents a significant improvement over previous methods.

Since the first submission of this work, another 36 eukaryotic signal sequences with known cleavage sites have been added to the data base. Using the same weight-matrix as above (Table 1), 75% of these sites were correctly predicted.

ACKNOWLEDGEMENT

This work was supported by a grant from the Swedish Natural Sciences Research Council.

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Article

Membrane protein structure prediction *1

Hydrophobicity analysis and the positive-inside rule

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Abstract

A new strategy for predicting the topology of bacterial inner membrane proteins is proposed on the basis of hydrophobicity analysis, automatic generation of a set of possible topologies and ranking of these according to the positive-inside rule. A straightforward implementation with no attempts at optimization predicts the correct topology for 23 out of 24 inner membrane proteins with experimentally determined topologies, and correctly identifies 135 transmembrane segments with only one overprediction.

Author Keywords: membrane protein; protein structure; prediction

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This Document
- **Abstract**
- Abstract + References
- PDF (1194 K)

Actions

1: Eur J Biochem. 1993 May 1;213(3):1333-40.

Predicting the topology of eukaryotic membrane proteins.

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We show that the so-called 'positive inside' rule, i.e. the observation that positively charged amino acids tend to be more prevalent in cytoplasmic than in extra-cytoplasmic segments in transmembrane proteins [von Heijne, G. (1986) EMBO J. 5, 3021-3027], seems to hold for all polar segments in multi-spanning eukaryotic membrane proteins irrespective of their position in the sequence and hence can be used in conjunction with hydrophobicity analysis to predict their transmembrane topology. Further, as suggested by others, we confirm that the net charge difference across the first transmembrane segment correlates well with its orientation [Hartmann, E., Rapoport, T. A. and Lodish H. F. (1989) Proc. Natl Acad. Sci. USA 86, 5786-5790], and that the overall amino-acid composition of long polar segments can also be used to predict their cytoplasmic or extra-cytoplasmic location [Nakashima, H. and Nishikawa, K. (1992) FEBS Lett. 303, 141-146]. We present an approach to the topology prediction problem for eukaryotic membrane proteins based on a combination of these methods.

MeSH Terms:

- Aspartic Acid/analysis
- Glutamates/analysis
- Glutamic Acid
- Membrane Proteins/analysis
- Membrane Proteins/chemistry*
- Research Support, Non-U.S. Gov't
- Tryptophan/analysis
- Tyrosine/analysis

Substances:

- Glutamates
- Membrane Proteins
- Tyrosine
- Aspartic Acid
- Glutamic Acid

TMpred - Prediction of Transmembrane Regions and Orientation

The TMpred program makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occuring transmembrane proteins. The prediction is made using a combination of several weight-matrices for scoring.

K. Hofmann & W. Stoffel (1993)
TMbase - A database of membrane spanning proteins segments
Biol. Chem. Hoppe-Seyler **374**,166

For further information see the TMbase and TMpredict documentation.

Usage: Paste your sequence in one of the supported formats into the sequence field below
and press the "Run TMpred" button.
Make sure that the format button (next to the sequence field) shows the correct format

Choose the minimal and maximal length of the hydrophic part of the transmembrane helix

Output format: html | minimum: 17 | maximum: 33

Query title (optional):

Input sequence format: Plain Text

Query sequence: or ID or AC or GI (see above for valid formats):

Run TMpred | Clear Input

Go back to the EMBnet.ch home page

MF C-35 A Database of Membrane Spanning Protein Segments

K. Hofmann and W. Stoffel
Institut fur Biochemie, Medizinische Fakultät, Universitat zu Köln, Köln, FRG

A database of all protein segments that are reported to span a membrane has been extracted from SwissProt 22. This sub-database consists of several tables that can be used with any relational database management system. The information stored within the database contains besides the sequence itself both annotational items extracted from SwissProt and additional data fields calculated from the sequence or taken from other sources. Important data fields include, for example, the putative transmembrane sequence, the sequence of the flanking regions, taxonomic information, the presumed orientation of the segment, calculated values for hydrophobicity and hydrophobic moment, and grouping into families by either functional or sequence relatedness of the proteins.
This database together with a set of related programs has been used to analyze the presumed transmembrane segments for positional preferences of amino acid residues. The influences of neighbouring residues, membrane protein classification, taxonomic classification and segment orientation on these positional preferences have been studied.

FEBS 18068

FEBS Letters 401 (1997) 163–166

Cloning of the human IL-13Rα1 chain and reconstitution with the IL-4Rα of a functional IL-4/IL-13 receptor complex

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Received 12 November 1996

Abstract The human homologue of the recently cloned murine IL-13 binding protein (IL-13Rα1) was cloned from a cDNA library derived from the carcinoma cell line CAKI-1. The cloned cDNA encodes a 427 amino acid protein with two consensus patterns characteristic of the hematopoietic cytokine receptor family and a short cytoplasmic tail. The human protein is 74% identical to the murine IL-13Rα1, and 27% identical to the human IL-13Rα2. CHO cells expressing recombinant hIL-13Rα1 specifically bind IL-13 ($K_d \approx 4$ nM) but not IL-4. Co-expression of the cloned cDNA with that of IL-4Rα resulted in a receptor complex that displayed high affinity for IL-13 ($K_d \approx 30$ pM), and that allowed cross-competition of IL-13 and IL-4. Electrophoretic mobility shift assay showed that IL-13 and IL-4 were able to activate Stat6 in cells expressing both IL-4Rα and IL-13Rα1, while no activation was observed in cells expressing either one or the other alone.

Key words: IL-13 binding protein; IL-13 signal transduction; IL-4 receptor complex

1. Introduction

Interleukin-13 (IL-13) is a cytokine secreted by activated T-lymphocytes which regulates inflammatory and immune responses [1,2]. It shares several biological activities with IL-4, another T-cell derived cytokine, in a variety of cell types such as B-cells, monocytes, fibroblasts and endothelial cells [3].

The functional redundancy of IL-4 and IL-13 suggested very early on that both cytokines probably shared receptor components [4–6]. The IL-4 receptor comprises two chains, the IL-4Rα and the γc [7–10]. Neither of these two chains binds IL-13 [5], but recent reports have shown that IL-4Rα contributes to the IL-13 receptor [11–13].

Recently, two proteins that bind specifically IL-13 have been cloned, one from murine tissue [14] and the other from human cells [15]. Since both proteins are most probably responsible for the initial interaction of IL-13 with the receptor complex(es) we propose to call them IL-13Rα1 and IL-13Rα2. IL-13Rα1 and IL-13Rα2 are distantly related (27% identity and 46% homology), but both proteins have short cytoplasmic domains, and two consensus patterns, four conserved cysteines in the amino-terminal half of the extra cellular domain and the WSXWS motif located in the C-terminal region of the extra cellular domain, considered signatures of the hematopoietic cytokine receptor family (for review see [19]). Interestingly, both proteins bind IL-13 with very different affinities, $K_d \approx 10$ nM and 50 pM for IL-13Rα1 and IL-13Rα2, respectively. We describe here the cloning of the human IL-13Rα1, and the pharmacological and functional characterization of the recombinant protein expressed alone or with IL-4Rα in stably transfected CHO cells.

2. Materials and methods

2.1. Growth factors and cells

Recombinant hIL-13 was produced and purified in our laboratory as previously described [2]. Human IL-4 was obtained from Tebu (Le Perray en Yvelines, France).

CAKI-1 cells (ATCC HTB 46), the B9 hybridoma cell line, and CHO cells were cultured as described [15].

2.2. cDNA library construction, isolation of cDNAs and sequence analysis

Total RNA from B9 hybridoma cells was used to synthesize cDNA [2]. A specific DNA fragment of the murine IL-13Rα1 was obtained by PCR using this cDNA and the following primers: 5'-AGAGGAATTACCCCTGGATG-3' (sense) and 5'-TCAAGGAGCTGCTGCTTTCTTCA-3' (anti-sense) corresponding to the nucleotides 249–268 and 1256–1275, respectively, of the mIL-13Rα1 sequence described by Hilton et al. [14].

The PCR product obtained (1027 bp) was purified, labelled (specific activity 2.4×10^9 dpm/μg) using the Random Primers DNA labelling kit (BRL), and used as a probe to screen a CAKI-1 cDNA library [15].

2.3. Binding and biological activity assays

Binding experiments on transfected CHO cells were performed using radiolabelled hIL-13 as described [5].

For the electrophoretic mobility shift assay (EMSA), 2×10^6 CHO cells or recombinant cell lines were plated onto 10 cm dishes and transfected 24 h later with 6 μg of plasmid DNA. After 48 h, the cells were washed and incubated in the presence of hIL-13 or hIL-4 (10 nM) for 30 min at 37°C, then rinsed twice with cold PBS containing 0.5 mM EDTA, harvested with a cell scraper in 1.2 ml PBS and finally transferred into 1.5 ml microcentrifuge tubes. Cellular extracts were prepared as described by Jiang and Eberhardt [16]. Gel shift assays were performed as described by Kölher et al. [17] with 10–20 μg of proteins and 5×10^4–1×10^5 cpm of the ^{32}P-labelled probe corresponding to the human Cε element from the human Cε control region [18] (5'-GATCCACTTCCCAAGAACAGA-3', the core sequence is underlined). Stat6 containing complexes were confirmed by supershifting with 2 μg of a monoclonal antibody anti-Stat6, M20 (Santa Cruz, CA), added to the binding reaction before EMSA.

3. Results

3.1. Cloning and sequencing of the human IL-13Rα1

A DNA fragment of the murine IL-13Rα1 [14] was derived from B9 total RNA and used to screen by hybridization a CAKI-1 cDNA library. Homologous sequences were relatively abundant (1/5000). The homologous full length cDNA is 3999 bases long, excluding the poly-A tract, and has a long 3' untranslated region of 2145 bases. A canonical AATAAA polyadenylation signal is found at the predicted location. The open reading frame between nucleotides 34 and 1851 defines a

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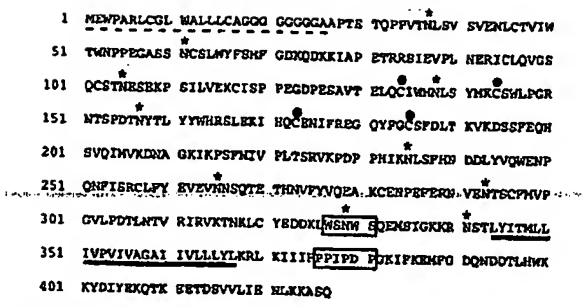

Fig. 1. Amino acid sequence of human IL-13Rα1. The amino acids corresponding to the predicted signal peptide are indicated with dashes. Potential *N*-glycosylation sites (Asn-X-Ser/Thr) are labelled with asterisks. Conserved cysteines in the hematopoietic cytokine receptor family are labelled with solid circles. The WSXWS and PXXPXP motifs are boxed. And the transmembrane domain is underlined. The human IL-13Rα1 cDNA sequence has been submitted to the EMBL Data Library (accession number Y09328).

polypeptide of 427 amino acids. The sequence codes for a membrane protein with a putative signal peptide, a single membrane-spanning domain and a short cytoplasmic tail (Fig. 1). Ten sites for potential N-linked glycosylation are located in the extracellular region. Importantly, two consensus patterns considered signatures of the hematopoietic cytokine receptor family (for review see [19]) are also found, four conserved cysteines in the amino-terminal half of the extra cellular domain, and the WSXWS motif located in the C-terminal region of the extra cellular domain. Furthermore, a proline-rich motif (PXXPXP) is located in the cytoplasmic region near the transmembrane domain. Alignment studies reveal homologies with the murine IL-13Rα1 (74% identity and 84% similarity) and to a lesser extent with the human IL-13Rα2 (27% identity and 51% similarity) and with the human IL-5Rα (26% identity and 46% similarity).

3.2. Expression and characterization of the IL-13 binding protein

CHO cells transfected with the isolated cDNA encoding the IL-13Rα1 showed specific binding of labelled IL-13. Scatchard analysis of the saturation curve showed a single component site with a K_d value of 4.5 ± 0.5 nM and a maximal binding capacity of 2.6×10^4 receptors/cell (Fig. 2A). The affinity displayed by the recombinant receptor is much lower than that displayed by the IL-13Rα2, with a K_d of 57 ± 10 pM [15]. However, when the saturation experiments were performed on CHO cells co-expressing IL-13Rα1 with IL-4Rα, the Scatchard analysis clearly showed the presence of two sites for IL-13 (Fig. 2B). One exhibited a dramatic increase in affinity (K_d: 32 ± 8 pM), and the other had a K_d similar to the one observed in the cells expressing IL-13Rα1 alone, 4.2 ± 1.4 nM. The high affinity binding site was not detected if the saturation experiments were performed in the presence of a large excess of IL-4 (not shown). No modification in IL-13 affinity resulted from the co-expression of IL-13Rα1 and IL-13Rα2 (not shown) and IL-13 did not bind to IL-4Rα, as previously described [5].

In competition studies, IL-13 was effective in inhibiting the labelled IL-13 binding to the cells expressing the IL-13Rα1. Labelled IL-4 neither bound to the IL-13Rα1, nor inhibited the binding of labelled IL-13 to this receptor (Fig. 3A). Several other cytokines (IL-2, IL-3, IL-5, IL-7, GM-CSF) were not able to displace IL-13 binding (not shown). However, when IL-13Rα1 and IL-4Rα were co-expressed in CHO cells a high affinity binding site for IL-13 was reconstituted, as shown in Fig. 2B, and this high affinity IL-13 binding was fully displaced not only by IL-13 but also by IL-4 (Fig. 3B). Co-expression of the IL-13Rα1 and IL-4Rα did not change the affinity of the IL-4 receptor for IL-4 (not shown) but allowed displacement of labelled IL-4 by IL-13 (Fig. 3C). These results show that both receptor chains interact in the cell membrane to reconstitute a receptor complex that displays high affinity for IL-13 and that is shared by both IL-13 and IL-4.

3.3. Biological activity

To examine whether IL-13Rα1 is able to transduce a signal to the cell we analyzed the activation of Stat6 because this regulator of gene transcription is activated by IL-13 and IL-4 [17]. Stable transfectants expressing IL-13Rα1 either alone or in combination with IL-4Rα were stimulated with IL-13 or IL-4 and the nuclear extracts were analyzed for binding to an oligonucleotide probe containing the Cε Stat response element from the Cε human control region [18]. The results (Fig. 4) showed that no activation was detected in non-transfected CHO cells incubated with IL-4 or IL-13. Similar negative results were observed on IL-4 or IL-13 stimulation of CHO cells expressing either IL-4Rα or IL-13Rα1. However, in CHO cells expressing both chains, IL-4Rα and IL-13Rα1, stimulation with IL-4 or IL-13 clearly resulted in a binding activity to the oligonucleotide probe in the nuclear extracts. The presence of Stat6 in the complexes was confirmed by supershifting experiments as described in Section 2 (not shown).

4. Discussion

We describe here the cloning and characterization of the human IL-13Rα1. The protein, homologous to the IL-13 binding protein recently cloned from murine tissue (IL-13Rα1) [14], recognizes IL-13 with much lower affinity than the other IL-13 binding protein cloned from human cells (IL-13Rα2) [15]. IL-13 binding to CHO cells expressing hIL-13Rα1 cannot be displaced by IL-4. Co-expression of IL-4Rα with IL-13Rα1 resulted in the reconstitution of a receptor complex that bound IL-13 with higher affinity than the IL-13Rα1 alone, and that allowed cross-competition between IL-

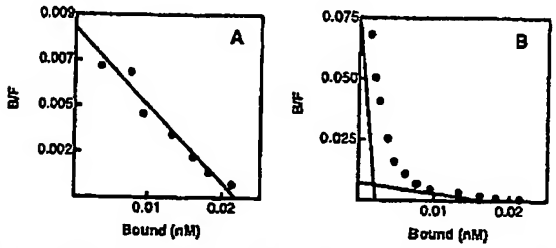

Fig. 2. Characterization of the recombinant IL-13Rα1 expressed in CHO cells. Scatchard analysis of the [^{125}I]IL-13 saturation curve with cells expressing (A) IL-13Rα1, which indicated the presence of ~26 000 sites/cell with a K_d of 4.5 ± 0.5 nM and (B) IL-13Rα1 and IL-4Rα, which indicated the presence of ~4000 sites/cell with a K_d of 32 ± 8 pM and of ~20 000 sites/cell with a K_d of 4.2 ± 1.4 nM.

13 and IL-4 as previously described for the murine IL-13Rα1 [14]. The experiments of activation of Stat6, as assayed by its property to bind to a specific sequence from the Cε promoter, complete and extend the binding results. IL-13Rα1 by itself is not capable of transducing a signal either for IL-13 or for IL-4, but when co-expressed with IL-4Rα it is capable of reconstituting a receptor complex that is able to transduce a signal for both cytokines. It should be noted that CHO cells expressing only IL-4Rα do not respond to IL-4 as measured by Stat6 activation. Since CHO cells do not express γc (unpublished results), the results are in line with previous reports that indicated the need for γc for the reconstitution of a functional IL-4 receptor [20]. The activation of Stat6 by IL-4 in cells co-expressing IL-4Rα and IL-13Rα1 clearly show that IL-13Rα1 can replace γc for the reconstitution of an active IL-4 receptor. The fact that IL-13Rα1 can replace γc for the reconstitution of an active IL-4 receptor explains, as previously suggested [6], the conflicting reports describing the need for γc for an active IL-4 receptor [9,20], and the description of active IL-4 receptors in the absence of γc [21,22]. Since the cytoplasmic domain of IL-13Rα1 is 26 amino acids shorter than that of γc we are currently investigating whether IL-13Rα1 contributes to the recruitment of Jak3, as described for γc [23], and/or to other signaling events as recently suggested [24]. In this context, it is important to emphasize the presence in the IL-13Rα1 of a proline-rich motif located in the cytoplasmic region near the transmembrane domain suggesting that IL-13Rα1 can associate with some kinases of the Jak family [25]. Together, these results show that IL-13Rα1 and IL-4Rα are sufficient to reconstitute a functional receptor for IL-13 and IL-4, and they do not exclude the possibility that other protein(s) may be associated in some cell types with the natural IL-4/IL-13 receptor complex as recently described for γc [24,26]. Two recent reports describe the homodimerization of IL-4Rα and, as a result, the intracellular signaling that finally leads to Stat6 activation. In both reports chimeric receptors were used in which the cytoplasmic and transmembrane domains of IL-4Rα were fused to the extracellular domain of the erythropoietin receptor [27] and γc [28], and dimerization was induced either with erythropoietin or with a monoclonal antibody. The apparent contradiction of these reports with our observation that CHO cells expressing IL-4Rα alone do not respond to IL-4 may indicate that if two IL-4Rα cytoplasmic domains are brought together they are able to transduce a signal to the cell, but that IL-4 does not induce dimerization of natural IL-4Rα. In line with this hypothesis are the results of Hoffman et al. who showed that IL-4 forms a 1:1 complex with the soluble portion of IL-4Rα [29]. Alternatively, the dimerization and activation of IL-4Rα by IL-4 may depend on the density of the receptor in the cell membrane, and/or on the presence of other subunit(s) of the receptor complex that are absent in CHO cells.

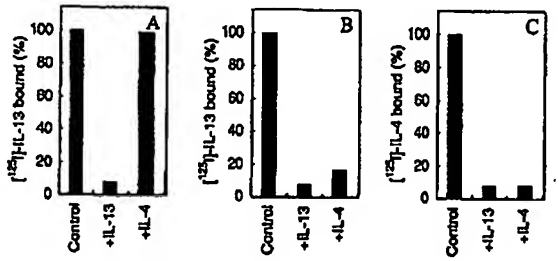

Fig. 3. Cross-competition of IL-13 and IL-4 on CHO cells expressing IL-13Rα1 alone or with IL-4Rα. A: Displacement of labelled IL-13 to cells expressing IL-13Rα1 by IL-13 (20 nM) and IL-4 (20 nM). B: Displacement of labelled IL-13 by IL-13 (20 nM) and IL-4 (20 nM) on cells expressing IL-13Rα1 and IL-4Rα. C: Displacement of labelled IL-4 by IL-13 (20 nM) and IL-4 (20 nM) on cells expressing IL-13Rα1 and IL-4Rα.

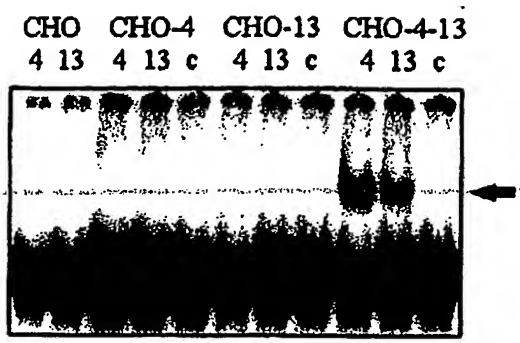

Fig. 4. Signal transduction of IL-13 and IL-4 in CHO cells expressing IL-13Rα1 alone or with IL-4Rα. The different cell lines, CHO, CHO expressing IL-4Rα (CHO-4), IL-13Rα1 (CHO-13), and IL-4Rα and IL-13Rα1 (CHO-4-13) were incubated in the absence (c) or in the presence of 5 nM of IL-4 (4) or IL-13 (13) as indicated and then the nuclear extracts were analyzed for Stat6 activation as described in Section 2.

In conclusion, our results demonstrate that IL-13Rα1 and IL-4Rα in the absence of γc are sufficient for the reconstitution of an active IL-13 and IL-4 receptor. The availability of the human IL-13Rα1 and IL-4Rα should allow the design of experiments to better assess the stoichiometry and the role played by each protein, and the relationship with γc and human IL-13Rα2, in the functional IL-4/IL-13 receptor complex.

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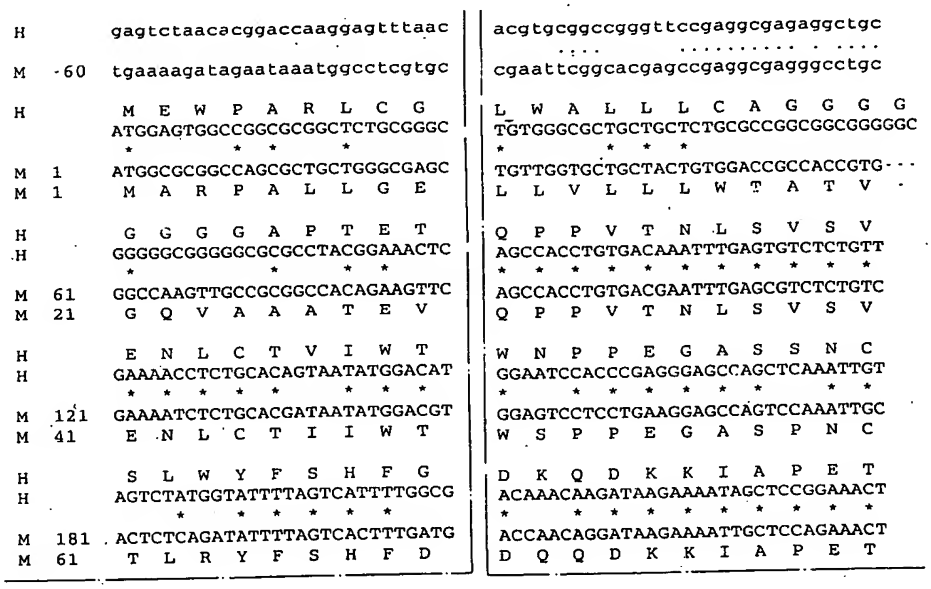

Fig. 7(i)

Fig. 7(ii)

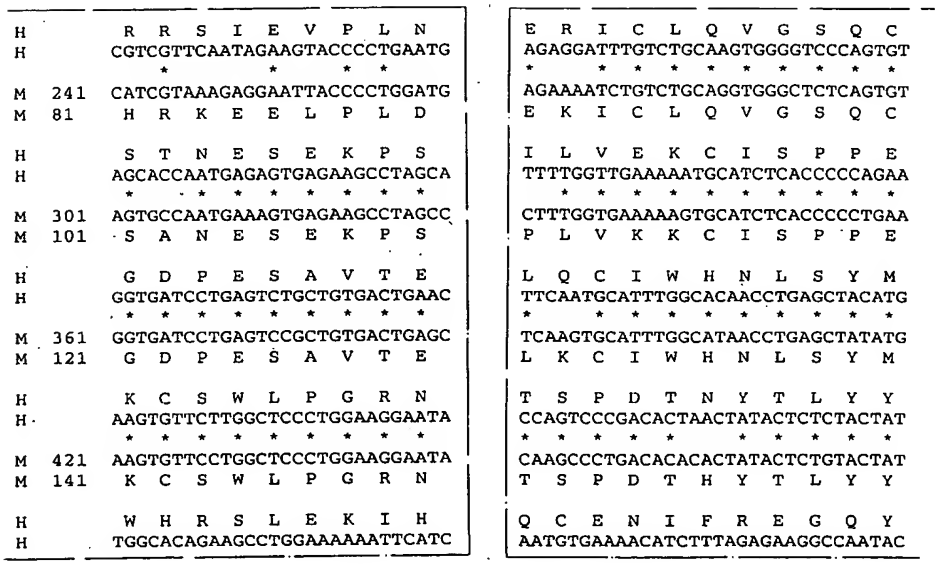

Fig. 7(iii)

Fig. 7(iv)

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          *       *  *  *  *
M  481  TGGTACAGCAGCCTGGAGAAAAGTCGTC
M  161   W  Y  S  S  L  E  K  S  R

H        F  G  C  S  F  D  L  T  K
H       TTTGGTTGTTCCTTTGATCTGACCAAAG
           *  *  *     *  *  *
M  541  ATTGCTTGTTCCTTTAAATTGACTAAAG
M  181   I  A  C  S  F  K  L  T  K

H        Q  I  M  V  K  D  N  A  G
H       CAAATAATGGTCAAGGATAATGCAGGAA
         *  *  *  *  *  *  *  *  *
M  601  CAAATAATGGTCAAGGATAATGCTGGGA
M  201   Q  I  M  V  K  D  N  A  G

H        T  S  R  V  K  P  D  P  P
H       ACTTCCCGTGTGAAACCTGATCCTCCAC
         *  *     *  *  *  *  *  *
M  661  ACTTCCTATGTGAAACCTGATCCTCCAC
M  221   T  S  Y  V  K  P  D  P  P

H        L  Y  V  Q  W  E  N  P  Q
H       CTATATGTGCAATGGGAGAATCCACAGA
         *     *  *  *     *  *  *
M  721  TTATTAGTGCAGTGGAAGAATCCACAAA
M  241   L  L  V  Q  W  K  N  P  Q
```

Fig. 7(v)


```
 *  *  *  *  *     *  *  *  *  *
AATGTGAAAACATCTATAGAGAAGGTCAACAC
Q  C  E  N  I  Y  R  E  G  Q  H

V  K  D  S  S  F  E  Q  H  S  V
TGAAGGATTCCAGTTTTGAACAACACAGTGTC
*           *  *  *           *
TGGAACCT---AGTTTTGAACATCAGAACG TT
V  E  P  -  S  F  E  H  Q  N  V

K  I  K  P  S  F  N  I  V  P  L
AAATTAAACCATCCTTCAATATAGTGCCTTTA
*  *     *  *        *  *     *
AAATTAGGCCATCCTGCAAAATAGTGTCTTTA
K  I  R  P  S  C  K  I  V  S  L

H  I  K  N  L  S  F  H  N  D  D
ATATTAAAAACCTCTCCTTCCACAATGATGAC
*  *  *     *        *
ATATTAAACATCTTCTCCTCAAAAATGGTGCC
H  I  K  H  L  L  L  K  N  G  A

N  F  I  S  R  C  L  F  Y  E  V
ATTTTATTAGCAGATGCCTATTTTATGAAGTA
*  *     *  *  *  *     *  *  *
ATTTTAGAAGCAGATGCTTAACTTATGAAGTG
N  F  R  S  R  C  L  T  Y  E  V
```

Fig. 7(vi)


```
H        E  V  N  N  S  Q  T  E  T
H       GAAGTCAATAACAGCCAAACTGAGACAC
         *  *  *  *     *  *
M  781  GAGGTCAATAATACTCAAACCGACCGAC
M  261   E  V  N  N  T  Q  T  D  R

H        E  N  P  E  F  E  R  N  V
H       GAGAATCCAGAATTTGAGAGAAATGTGG
           *     *        *  *
M  841  CAGAATTCCGAATCTGATAGAAACATGG
M  281   Q  N  S  E  S  D  R  N  M

H        L  P  D  T  L  N  T  V  R
H       CTTCCTGATACTTTGAACACAGTCAGAA
         *     *           *  *  *
M  901  CTTGCCGACGCTGTCTACACAGTCAGAG
M  301   L  A  D  A  V  Y  T  V  R

H        D  D  K  L  W  S  N  W  S
H       GATGACAAACTCTGGAGTAATTGGAGCC
         *     *  *  *  *     *  *
M  961  GACAACAAACTGTGGAGTGATTGGAGTG
M  321   D  N  K  L  W  S  D  W  S

H        T  L  Y  I  T  M  L  L  I
H       ACACTCTACATAACCATGTTACTCATTG
```

Fig. 7(vii)


```
H  N  V  F  Y  V  Q  E  A  K  C
ATAATGTTTTCTACGTCCAAGAGGCTAAATGT
*  *           *     *     *  *
ATAATATTTTAGAGGTTGAAGAGGACAAATGC
H  N  I  L  E  V  E  E  D  K  C

E  N  T  S  C  F  M  V  P  G  V
AGAATACATCTTGTTTCATGGTCCCTGGTGTT
*     *  *  *  *        *  *  *
AGGGTACAAGTTGTTTCCAACTCCCTGGTGTT
E  G  T  S  C  F  Q  L  P  G  V

I  R  V  K  T  N  K  L  C  Y  E
TAAGAGTCAAAACAAATAAGTTATGCTATGAG
   *  *  *  *  *  *  *  *
TAAGAGTCAAAACAAACAAGTTATGCTTTGAT
V  R  V  K  T  N  K  L  C  F  D

Q  E  M  S  I  G  K  K  R  N  S
AAGAAATGAGTATAGGTAAGAAGCGCAATTCC
         *  *  *  *        *  *
AAGCACAGAGTATAGGTAAGGAGCAAAACTCC
E  A  Q  S  I  G  K  E  Q  N  S

V  P  V  I  V  A  G  A  I  I  V
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```

Fig. 7(viii)

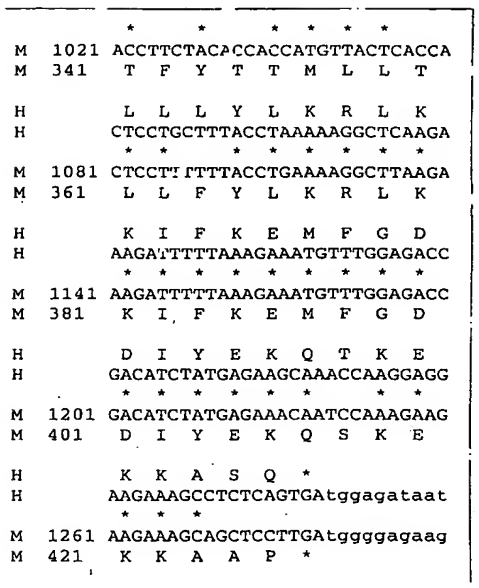

Fig. 7(ix)

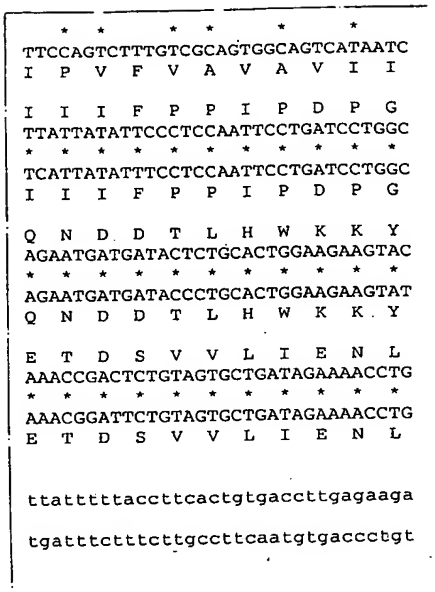

Fig. 7(x)